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In re application of: HAMMERMAN, *et al.*	Examiner: F. Moezie
	Group Art Unit: 1653
Serial No.: 09/222,460	
Filed: December 29, 1998	
For: COMPOSITION AND METHOD FOR IMPROVING FUNCTION OF EMBRYONIC KIDNEY TRANSPLANTS	

CERTIFICATE OF MAILING

I hereby certify that this correspondence, including listed enclosures, is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231 on October 2, 2001

Signed: Mary M Fuld

DECLARATION UNDER 37 C.F.R. §1.132

I, Marc R. Hammerman, do declare and state as follows:

1. I am a citizen of the United States of America and reside in St. Louis, Missouri.

2. Sharon A. Rogers and I are the named co-inventors of the above-identified application. However, I am the sole inventor of the subject matter claimed relating to the growth factor treatment of metanephric tissue at the time of or after transplantation into a recipient. Ms. Rogers and I are co-inventors of the subject matter claimed relating to the growth factor treatment of metanephric tissue prior to transplantation into a recipient and at the time of ureteroureterostomy.

3. I have reviewed and am familiar with the reference EP 0 853 942. Consistent with the foregoing paragraph 2, I am the sole inventor of the subject matter in that European patent application. In connection with the subject matter described in the European patent application, I conceived the idea of treating metanephric tissue with growth factors at the time of and/or after transplantation continuously, for example, by subcutaneous osmotic pumps. Sharon A. Rogers did not contribute to the conception of that invention.

4. As indicated, Sharon A. Rogers and I are co-inventors of the method of pretreatment of metanephric tissue with growth factors before transplantation and the method of treating implanted

metanephric tissue by bathing it in growth factors-containing solution at the time of ureteroureterostomy. The pretreatment of metanephric tissue with growth factor and treatment of implanted metanephric tissue with growth factor at the time of ureterorooureterostomy resulted in unexpected positive effects on the implanted tissue. For instance, Example 6 of this application shows significantly improved function of the implanted metanephroi with VEGF treatment at the time of ureteroureterostomy as compared to no treatment (page 18, lines 24-30). Example 7 of this application (Table 5, p. 19) shows significantly improved function of the implanted metanephroi by pretreatment with a mixture of growth factors (page 19, lines 2-3) as compared to no treatment. Further, Exhibit A is a copy of one of my publications relating to this subject matter (Hammerman, Marc R., "Transplantation of renal precursor cells: a new therapeutic approach," Pediatr. Nephrol. 14:513-517, 2000). As shown in Table 1 (columns 1 and 2) at page 516, pretreatment with VEGF alone resulted in improved function of the implanted tissue. Improved function of the implanted tissue by treating the tissue with a growth factor for 45 minutes at the time of ureteroureterostomy is set forth at column 3 in Table 1.

5. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9/21/01

Marc R. Hammerman

Pediatr Nephrol (2000) 14:513–517

DEVELOPMENTAL BIOLOGY REVIEW

EXHIBIT

A

Marc R. Hammerman

Transplantation of renal precursor cells: a new therapeutic approach

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Abstract The number of kidney transplantations performed per year is limited due to availability of donor organs. One possible solution to the organ shortage is the use of renal xenografts. However, the transplantation of xenografts is complicated by hyperacute and acute rejection. It has been postulated that the host immune response might be attenuated following the transplantation of renal precursor cells or embryonic kidneys (metanephroi) instead of developed (adult) kidneys. Transplanted metanephroi become chimeric organs in that their blood supply originates, at least in part, from the host. It is possible to transplant a developing metanephros, without the use of immunosuppression, from one rat to another. Transplanted metanephroi grow, develop, become vascularized, and function in host rats. Transplantation of metanephroi may be a promising novel therapeutic approach for the treatment of chronic renal failure.

Key words Allograft · Metanephros · Transplantation · Xenograft

Introduction

End-stage chronic renal failure afflicts more than 300,000 individuals in the United States alone, most of whom are treated using dialysis, a treatment with considerable morbidity [1], or renal allotransplantation, which is limited by the number of organs available for transplantation [2]. One possible solution to the lack of organ availability is the use of renal xenografts. The clinical renal xenografts performed to date have utilized primate donors, because the closer species are phylogenetically, the more easily the xenografts are accepted [3]. Clinical experience with the use of primates as kidney donors dates from the 1960s. However, the results of xenografting kidneys have been unsatisfactory, and this technique has remained an experimental one [3].

A second possible solution to the lack of organ availability is the transplantation of renal precursor cells or developing kidneys (metanephric allografts or xenografts). The allotransplantation of renal precursors or developing metanephroi into adult animals has been performed by several investigators [4, 5, 6, 7, 8, 9, 10, 11, 12, 13].

There are four theoretical reasons why the transplantation of renal precursor cells or developing kidneys (metanephroi) into adult animals might be advantageous relative to the transplantation of developed kidneys. First, if obtained at a sufficiently early stage, such as prior to embryonic day 15 (E15) in the rat, a developing metanephros would be expected to be depleted of dendritic antigen-presenting cells (APCs) [14] that mediate "direct" host recognition of alloantigen or xenoantigen [15]. Dendritic cells would be absent from developing metanephroi by virtue of the absence of a vasculature from which APCs can enter the organ [10] and the absence of mature APCs themselves at this stage of rat development [14].

It has been suggested that APC depletion from rat pancreatic islets, resulting in prolongation of islet survival after injection into the portal vein of host mice, can be achieved by culturing the islets in vitro prior to injection [16].

It is more difficult to achieve the same results with whole vascularized organs [17,18]. Dendritic cells can be depleted from developed kidneys by the combination of total body irradiation and cyclophosphamide pretreatment of donors. APC depletion in this manner prolongs graft survival in MHC (RT1) incompatible rat hosts [18]. Also, long-surviving "immunologically enhanced" (den-

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dritic cell-depleted) kidneys transplanted into MHC incompatible hosts do not elicit strong primary T cell-dependent alloimmunity after transplantation into a secondary recipient of the same genotype as the original host [17]. "Immunological enhancement" is accomplished by injecting hosts with donor-strain spleen cells prior to transplantation and host anti-donor antiserum prior to transplantation and at the time of transplantation [17].

Secondly, the transplanted metanephros becomes a chimeric organ in that it is vascularized in part by blood vessels originating from the host [4, 10]. Hyperacute rejection of xenografts, which is initiated by circulating preformed natural antibodies that activate complement after binding to glycoprotein antigens on endothelial cell surfaces [19, 20], should be circumvented to the extent that the transplanted organ is supplied by host vessels [3].

Insight into the origin of the renal blood supply is provided by experiments in which developing kidneys are transplanted to ectopic sites. In the case of 11-day-old mouse or chick metanephroi grafted onto the chorioallantoic membrane of the quail, the vasculature is derived entirely from the host [11]. In the case of 11- to 12-day-old mouse metanephroi grafted into the anterior chamber of the eye, the glomerular microvascular endothelium derives from both donor and host [5]. In either case, large external vessels derive from the host [5, 11].

Thirdly, the expression of class II MHC heterodimers on non-lymphoid, renal proximal tubule cells without co-expression of co-stimulatory receptors such as B7 [21], may serve as an extra thymic mechanism for the maintenance of immune tolerance.

Fourthly, the trafficking of host T cells through transplanted neonatal non-lymphoid tissue may represent a mechanism by which hosts can be rendered tolerant to that tissue [22].

Transplantation of developing metanephroi into host kidneys

The possibility that renal function can be enhanced through the addition of functioning nephrons via transplantation of metanephroi or renal precursor cells into functioning kidneys has been explored.

Woolf et al. [6] implanted pieces of sectioned metanephroi originating from E13–E16 mice into tunnels fashioned in the cortex of kidneys of newborn outbred mice. Differentiation and growth of donor nephrons occurred in the host kidney. Glomeruli were vascularized, mature proximal tubules were formed, and extensions of metanephric tubules into the renal medulla were observed. Glomerular filtration was demonstrable in donor nephrons using fluorescently labelled dextran as a marker of filtration into the proximal tubules. However, connection of donor nephrons to the collecting system of hosts, which would be required for plasma clearance, could not be demonstrated. In contrast to the case in newborn mice, metanephric tissue transplanted into kidneys of adult mice neither grew nor differentiated, but was extruded as a mass under the renal capsule, resembling a poorly differentiated tumor. It was concluded that the neonatal kidney, which has a rim of undifferentiated cortex (the nephrogenic zone), can facilitate the differentiation of an embryonic implant, but that this ability is lacking in the fully differentiated adult kidney [6].

Abrahamson et al. [5] implanted metanephroi from E17 rat embryos beneath the renal capsule of five adult rat hosts. Within 9–10 days after implantation, every graft became vascularized, new nephrons were induced to form, and glomerular and tubular cytodifferentiation occurred. Glomeruli from transplanted metanephroi were identifiable because they were approximately two-thirds the diameter of those within host kidneys. Intravenous injection of anti-laminin IgG into hosts resulted in labelling of glomerular basement membranes of grafted kidneys, confirming perfusion of the grafts by the hosts' vasculature [5].

Robert et al. [7] grafted metanephroi from E12 mouse embryos into kidney cortices of adult and newborn ROSA26 mouse hosts. ROSA26 mice bear a ubiquitously expressed β-galactosidase transgene that can be identified by staining in histological sections, permitting differentiation of transplanted from host tissue. Grafts into both newborn and adult hosts examined 7 days post transplantation were vascularized by components originating from both donor and host [7].

Koseki et al. [8] transplanted rat nephrogenic mesenchymal cells that had been transfected with a *Lac Z* reporter gene by a retrovirus underneath the capsule of kidneys of neonatal rats. Transplanted mesenchymal cells were integrated into functioning host nephron segments.

We transplanted E15 rat metanephroi beneath the renal capsule of adult Sprague-Dawley rat kidneys. Within several weeks transplanted metanephroi had developed glomeruli, and mature proximal and distal tubules characteristic of mature nephrons. In addition, collecting ducts of donor origin grew towards the papilla of the host [9]. However, as in the studies of Woolf et al. [6], we could not demonstrate any connection between the collecting systems of the donor and host kidneys [9].

When host kidneys were examined 4 or 6 weeks following renal subcapsular transplantation, cysts containing clear fluid surrounded the sites where metanephroi were transplanted under the renal capsule [9]. Levels of urea nitrogen and creatinine were measured in aspirated cyst fluid, in blood from the aorta, and urine from the bladder of rats that had received subcapsular metanephric implants. Levels of urea nitrogen were increased 2.6-fold and 15-fold, respectively in cyst fluid and bladder urine relative to blood, and levels of creatinine were increased 12-fold and 28-fold, respectively. Thus, both urea nitrogen and creatinine were concentrated in cyst fluid relative to blood. The concentrations of urea nitrogen and creatinine in cyst fluid were significantly less than the concentrations in bladder urine [9]. These obser-

vations raise the possibility that the cyst fluid represents urine originating from the transplant. The dilution of cyst fluid relative to bladder urine is consistent with the reduced ability of a 4-week-old kidney (transplanted kidney) to clear the blood of urea nitrogen and creatinine relative to a 10-week-old kidney (host kidney) [9].

Armstrong et al. [12] previously reported the formation of cysts in metanephroi transplanted under the kidney capsule of mice. They suggested that the presence of cysts in developed donor metanephroi, coupled with their inability to demonstrate any connection between the donor and host collecting systems, raised the possibility that transplanted metanephroi become obstructed in the subcapsular site. Our findings [9] are consistent with theirs [12]. In addition, growth of metanephroi transplanted under the renal capsule may be physically constrained by their location between host renal parenchyma and the host capsule.

Barakat and Harrison [13] had shown that sections of rat metanephroi transplanted into a subcutaneous site in the abdominal wall of host rats remained vascularized for several days, but were replaced by fibrosis within 2–3 weeks. To reduce constraints to metanephroi growth, render obstruction less likely, and assure connection to the host collecting system, we investigated the feasibility of transplanting metanephroi into the host's omentum (intra-abdominally), sufficiently near to one of the ureters to render possible ureteroureterostomy between transplant and host [9].

Transplantation of developing metanephroi into the omentum

We implanted whole metanephroi from E15 outbred Sprague-Dawley rats into the omentum of non-immunosuppressed adult Sprague-Dawley rat hosts [9]. At the time of implantation, some host rats underwent unilateral nephrectomy (UNX) or unilateral nephrectomy and partial contralateral renal infarction (1 1/2 NX). E15 metanephroi contained only condensed metanephric blastema and segments of ureteric bud.

Metanephroi from E15 rats had enlarged 4–6 weeks post implantation, had become vascularized, and had formed mature tubules and glomeruli. Ureters of metanephroi transplanted into the omentum were anastomosed to hosts' ureters that remained after UNX. Four weeks following ureteroureterostomy, the contralateral kidney was removed. Transplanted metanephroi were shown to clear inulin infused into the hosts' circulation.

The transplanted metanephroi were vascularized by arteries originating from the omentum. Both weights of transplanted metanephroi and inulin clearances of transplanted metanephroi were significantly increased in rats that underwent 1 1/2 NX compared with UNX. In contrast, transplantation of developed kidneys resulted in rejection.

Electron microscopy of metanephroi transplanted 12 weeks previously into UNX rats was performed [23] (Fig. 1). A glomerular capillary loop is shown in Fig. 1a. A mesangial cell is labelled (*m*). A higher-power view of another glomerular capillary loop with an endothelial cell labelled (*en*) is shown in Fig. 1b. A still higher-power view showing an epithelial cell (*ep*), an endothelial cell (*en*), podocytes (*pd*), and a basement membrane of normal thickness and appearance (*arrows*) is shown in Fig. 1c. A proximal tubule (*pt*) with a brush border membrane (*arrowhead*) is labelled in Fig. 1d. A proximal tubule (*pt*), distal tubule (*dt*), and collecting duct (*cd*) are labelled in Fig. 1e.

Our findings [9] establish: (1) that functional chimeric kidneys develop from metanephroi transplanted into adult hosts and (2) that the stimulus for compensatory renal growth enhances the growth of transplanted metanephroi.

The use of growth factors to enhance the growth and function of transplanted metanephroi

Experiments utilizing metanephric organ culture have shown that kidney development in vitro is dependent upon the expression of a number of polypeptide growth factors within the developing organ. Blocking the expression or action of any of the growth factors, transforming growth factor-α, hepatocyte growth factor, insulin-like growth factor I (IGF I) or insulin-like growth factor II (IGF II), inhibits growth and development in vitro [24, 25]. Vascular endothelial growth factor (VEGF) is also produced by developing kidneys. Blocking VEGF activity in vivo inhibits renal vascularization [26].

To investigate whether growth factors could be used to enhance the growth and function of developing metanephroi, we performed two types of experiments. First, we determined whether the administration to transplanted hosts of IGF I, a growth factor known to enhance renal growth and function in a variety of settings [24], could increase inulin clearance of transplanted metanephroi.

In preliminary experiments, we demonstrated that neither weights nor inulin clearances of metanephroi transplanted into UNX rats increased between weeks 12 and 32 post transplantation [24]. However, infusion of IGF I into hosts enhanced inulin clearances of metanephroi measured at 12–16 weeks post transplantation [24].

Secondly, we determined whether exposure of metanephroi to exogenous VEGF enhanced their growth and development. We exposed metanephroi to VEGF in two ways: (1) for 45 min prior to implantation into UNX host rats, E15 rat metanephroi were incubated at 4°C in 25 µl of a 50:50 mixture of Dulbecco's modified Eagles medium:Hams F12 (DMEM:HF12) containing 25 µg of recombinant human VEGF (Genentech, S. San Francisco, Calif., USA) or DMEM:HF12 containing no additions (pretreatment); or (2) for 45 min at 4 weeks post transplantation at the time of ureterouretostomy between the transplanted metanephros and the host, we bathed metanephroi in 25 µl of the 50:50 mixture of DMEM:HF12

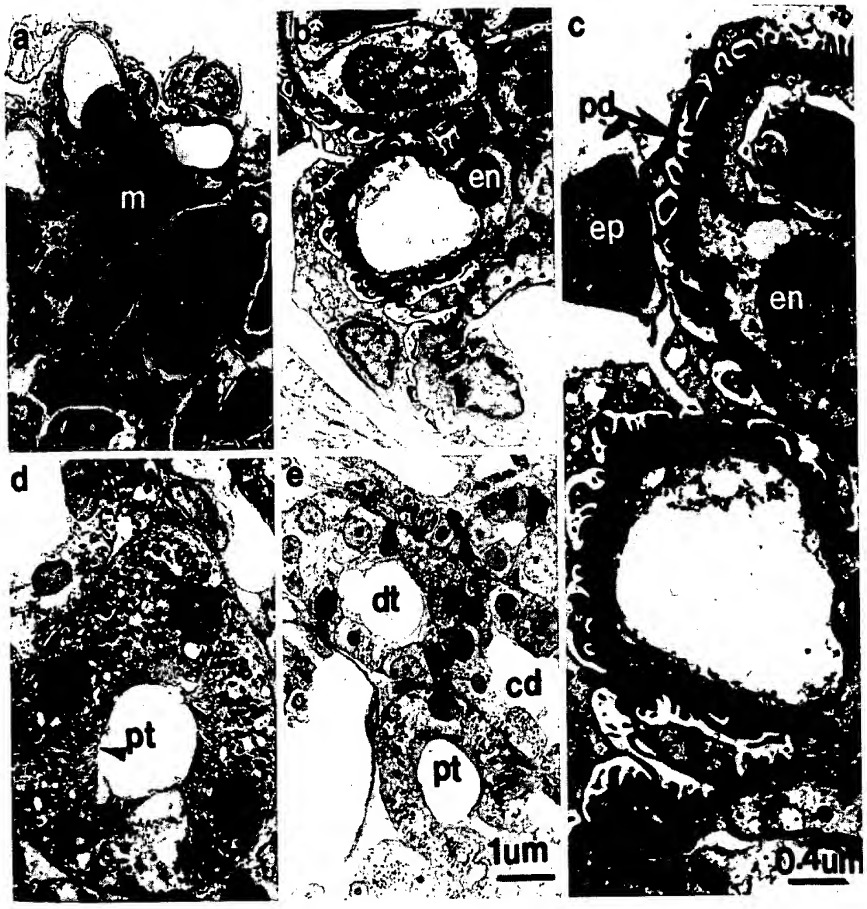

Fig. 1a–e Electron micrographs of transplanted rat metanephroi. **a** A glomerular capillary loop. A mesangial cell is labelled (*m*). **b** A glomerular capillary loop. An endothelial cell (*en*) is labelled. **c** A glomerular capillary loop. An epithelial cell (*ep*), an endothelial cell (*en*), and podocytes (*pd*) are labelled and a basement membrane is delineated (*arrows*). **d** A proximal tubule (*pt*) with a brush border membrane (*arrowhead*). **e** A proximal tubule (*pt*), distal tubule (*dt*), and collecting duct (*cd*). Magnifications are shown for **c** and **e**. Reprinted with permission [23]

containing 25 μg of recombinant human VEGF (post-treatment).

Treatment with VEGF did not affect the weights of transplanted metanephroi measured at 12–16 weeks following transplantation into UNX rats, compared with the weights of untreated metanephroi. However inulin clearances and urine volumes were increased significantly in metanephroi pretreated with VEGF, and increased further in post-treated metanephroi (Table 1).

Subsequently, using combinations of growth factors to pretreat and post-treat metanephroi, we have achieved rates of inulin clearance in transplanted metanephroi almost 300 times those measured in our first experiments [9], approximately 5% of the clearance achieved by a normal rat kidney [25].

Table 1 Weights of metanephroi, urine volumes, and inulin clearances[a]

	Implant (n=7)	VEGF pretreatment (n=6)	VEGF post-treatment (n=3)
Weight (μg)	72±10	75±8.5	81±17
Urine volume (μl/h)	31±9.1	74±7.5*[1]	184±26*[3,4]
Inulin clearance (μl/min per 100 g)	0.23±0.06	0.70±0.09*[1]	1.6±0.37*[2,5]

Implant<VEGF: *[1]P<0.05; *[2]P<0.01; *[3]P<0.001
VEGF pretreatment<VEGF post-treatment: *[4]P<0.01; *[5]P<0.001
[a] Data are expressed as mean±SEM. Multiple comparisons were performed using Bonferroni's test

Future directions

The studies described above demonstrate the feasibility of transplantation of allograft metanephroi subcapsularly in the kidney and into the omentum of adult outbred rat hosts. When implanted in the omentum, metanephroi grow, differentiate into renal structures with normal structure [9, 24] and ultrastructure (Fig. 1), become vascularized [9], and clear inulin from the circulation for as long as 32 weeks in vivo [24]. In contrast, transplanted adult kidneys are rejected within a week [9]. Studies performed concurrently demonstrated the feasibility of transplanting mouse metanephroi into the rat [25]. Investigations are being carried out using inbred strains of rats to determine why transplanted metanephroi are not rejected.

Inulin clearances in transplanted metanephroi are very low [9]. However, clearances can be enhanced by reduction of host native renal mass [9], by infusion of IGF I into hosts [24], and by exposure of metanephroi to the growth factor VEGF (Table 1). Future investigations will be designed to test whether combinations of growth fac-

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OCCASIONAL SURVEY

Bernard S. Kaplan · Karen Polise

In defense of altruistic kidney donation by strangers

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Abstract A shortage of cadaveric donor kidneys has created waiting lists for patients on chronic dialysis. Despite many ethical issues, donor kidneys are obtained from cadavers, first-degree living relatives, second-degree relatives (uncles, aunts), emotionally related persons such as spouses, and non-genetic altruistic donors who have a close relationship with the recipient. Most centers do not accept kidneys from minors, persons who have no genetic or personal relationship with the recipient, organs offered by altruistic strangers, or those that are purchased. The pros and cons of using kidneys from donors who are altruistic strangers (donors who have no genetic or personal relationship with the recipient) are reviewed. It may seem that organ acquisition for renal transplantation has moved down a slippery slope from cadaver donors to living non-related but emotionally related donors. However, it can also be argued that the approach to obtaining kidneys has evolved with improvements in safety to the donor and an increasing shortage of organs. It may also be argued that the approach should evolve from a paternalistic physician-centered role to a position in which the patient has more autonomy in deciding whether or not to accept a kidney from an altruistic donor.

Key words Renal transplant · Altruistic donor · Ethics · Living non-related donor

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Introduction

The purpose of this commentary is to explore some of the ethical issues that arise from the use of altruistic strangers as living non-related renal allograft donors. The terms, living non-related donor and altruistic donor, cannot always be used interchangeably. An *'emotionally-related living kidney donation'* is defined as the donation of a kidney by a non-blood-relative donor to a recipient with whom the donor is emotionally closely linked, has a credible motive, and does not accept a financial or material reward [1]. Unrelated transplants with organs from *'altruistic donors'* outside the family circle is a very different story according to Jakobsen [2]. Jakobsen defended the use of spousal kidney donation but eschewed the option of altruistic non-spousal donation without explaining his enigmatic comment that "such a practice may create more problems than it was meant to solve" [2]. That there may be profound ethical complexities and dilemmas arising from the use of altruistic donors is as certain a possibility as were the predictions at the dawn of organ transplantation that there would be profound ethical complexities and dilemmas associated with transplantation. Those predictions, many of which came true, fortunately did not prevent transplants from being performed.

Live-unrelated transplantation is a subject of great controversy [3]. Thomas and his colleagues have stated that "most of us feel that organ donation depends on the altruistic nature of people giving the ultimate gift and do not believe in donating organs for financial gain because it could lead to exploitation, blackmail, and emotional and financial problems. However, the assumption that all unrelated donors are paid ones is not necessarily true, because a stranger may genuinely want to repay a debt to humankind and relatives' motives for donation may not be altruistic. Can we therefore judge people's motives and get it right every time?" [3]. The reason that many oppose the acceptance of kidneys from non-related altruistic donors may in part be because of an almost visceral fear that this is only a short step from commercialization

tors to which metanephroi or hosts are exposed can further enhance inulin clearance of transplants.

Finally, although the rat provides a relatively inexpensive model with which to characterize the immunology, vascularization, differentiation, and growth of transplanted metanephroi, the applicability of this technique to humans will require that its feasibility be demonstrated in a larger mammal. Therefore, studies will be undertaken using the pig, a mammal larger than the rat, the kidneys of which are more similar to those of man, and which has been proposed as a potential source for xenografts for humans [27].

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